Journal of Environmental Management 95 (2012) S332–S337

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Characterization of rhizosphere bacteria for control of phytopathogenic fungi of tomato

Nicolás Pastor [a,*], Evelin Carlier [a], Javier Andrés [b], Susana B. Rosas [a], Marisa Rovera [c]

[a] Laboratorio de Interacción Microorganismo-Planta, Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Ruta 36, Km 601, CP X5804BYA, Río Cuarto, Córdoba, Argentina
[b] Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, Ruta 36, Km 601, CP X5804BYA, Río Cuarto, Córdoba, Argentina
[c] Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36, Km 601, CP X5804BYA, Río Cuarto, Córdoba, Argentina

ARTICLE INFO

Article history:
Received 15 August 2009
Received in revised form
3 March 2011
Accepted 29 March 2011
Available online 20 April 2011

Keywords:
Fluorescent *Pseudomonas* spp.
Antagonistic bacteria
Phytopathogenic fungi
Tomato

ABSTRACT

Fluorescent *Pseudomonas* spp., isolated from rhizosphere soil of tomato and pepper plants, were evaluated *in vitro* as potential antagonists of fungal pathogens. Strains were characterized using the API 20NE biochemical system, and tested against the causal agents of stem canker and leaf blight (*Alternaria alternata* f. sp. *lycopersici*), southern blight (*Sclerotium rolfsii* Sacc.), and root rot (*Fusarium solani*). To this end, dual culture antagonism assays were carried out on 25% Tryptic Soy Agar, King B medium, and Potato Dextrose Agar to determine the effect of the strains on mycelial growth of the pathogens. The effect of two concentrations of $FeCl_3$ on antagonism against *Alternaria alternata* f. sp. *lycopersici* was also tested. In addition, strains were screened for ability to produce exoenzymes and siderophores. Finally, the selected *Pseudomonas* strain, PCI2, was evaluated for effect on tomato seedling development and as a potential candidate for controlling tomato damping-off caused by *Sclerotium rolfsii* Sacc., under growth chamber conditions. All strains significantly inhibited *Alternaria alternata* f. sp. *lycopersici*, particularly in 25% TSA medium. Antagonistic effect on *Sclerotium rolfsii* Sacc. and *Fusarium solani* was greater on King B medium. Protease was produced by 30% of the strains, but no strains produced cellulase or chitinase. Growth chamber studies resulted in significant increases in plant stand as well as in root dry weight. PCI2 was able to establish and survive in tomato plants rhizosphere after 40 days following planting of bacterized seeds.

1. Introduction

Tomato (Lycopersicon esculentum Mill.) is the second leading vegetable crop worldwide, next to potato. World production is $\sim 1 \times 10^6$ tonnes from 3.7×10^6 ha (Food Agricultural Organization, 2010). In Argentina, it is the vegetable occupying the most greenhouse area. The percentage of total production going to industry is 35–40% and the rest is sold as fresh produce domestically. The area dedicated to tomato in field and greenhouse is 1.2×10^4 ha and 3×10^3 ha, respectively; average yield in both cases is ~35–40 tonnes per ha (Nakama and Fernández Lozano, 2006). Due to increasing demand, tomato has a great potential for increased commercialization. More efficient tomato production requires better knowledge of its pathogens and control methods.

The fungus *Alternaria alternata* f. sp. *lycopersici*, frequently isolated from diseased tomato plants, is the cause of stem canker (Gilchrist and Grogan, 1975) and leaf blight (Akhtar et al., 2004). *Sclerotium rolfsii* Sacc. is a soilborne fungus that causes southern blight disease in a wide variety of agricultural and horticultural crops (Flores-Moctezuma et al., 2006). *Fusarium solani* causes root rot in several crops. Penconazole [1-(2,4-dichloro-β-propylphenethyl)-1*H*-1,2,4-triazole], penthachloronitrobenzene (PCNB), and idropione [3-(3,5-dichlorophenyl)-N-(1-methylethyl)2,4-dioxo-1-imidazole-carboxamide] are three chemical fungicides commonly used to control the above pathogenic fungi. Nevertheless, increasing public concern regarding use of chemical pesticides that damage human health or the environment is driving the search for more environmentally "friendly" methods to control plant disease. A realistic alternative, or supplement, to chemical fungicides for management of plant diseases is the use of soilborne, non-

* Corresponding author. Tel.: +54 3584676103/232; fax: +54 3584676232.
E-mail addresses: npastor@exa.unrc.edu.ar (N. Pastor), ecarlier@exa.unrc.edu.ar (E. Carlier), jandres@ayv.unrc.edu.ar (J. Andrés), srosas@exa.unrc.edu.ar (S.B. Rosas), mrovera@exa.unrc.edu.ar (M. Rovera).

0301-4797/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2011.03.037

pathogenic bacteria that inhibit fungal phytopathogens. Such bacteria are known by several generic names, including "biological control agents" (BCAs) and "plant growth promoting rhizobacteria" (PGPR). Soilborne, fluorescent pseudomonads have received particular attention because of their catabolic versatility, excellent root-colonizing abilities, and production of a wide range of antifungal metabolites (Walsh et al., 2001).

The objectives of this study were (1) to biochemically characterize fluorescent *Pseudomonas* strains, (2) to evaluate their antagonistic activities against phytopathogenic fungi of vegetables *in vitro*, and (3) to determine the effect of a strain, PCI2, on tomato growth as well as to evaluate its potential for controlling tomato damping-off caused by *S. rolfsii* Sacc.

2. Materials and methods

2.1. Isolation and characterization of fluorescent Pseudomonas

Fluorescent *Pseudomonas* spp. were isolated from the rhizosphere of healthy tomato (*L. esculentum* Mill.) and pepper (*Capsicum annuum* L.) plants from four regions of the province of Córdoba, Argentina: Colonia Caroya (20° 36′ N, 102° 13′ W), Embalse (32° 12″ S, 64° 23″ W), Mattaldi (34° 49′ 16″ S, 64° 34′ 22″ W) and Río Cuarto (33° 04′ S, 64° 38′ W). Non rhizosphere soil was removed from the root system of the plants. Roots were then excised and placed into 10 ml of sterile 0.9% NaCl solution and vortexed for 10 min in order to detach the associated rhizosphere soil. Serial dilutions of the resulting root wash were plated on King B medium (KB) (King et al., 1954) supplemented with ampicillin (100 μg ml^{-1}) and cycloheximide (75 μg ml^{-1}) (Simon and Ridge, 1974). Plates were incubated at 28 °C for 24–48 h, at which time the fluorescent colonies were observed under UV light (354 nm). To obtain the most abundant bacteria from each sample, selection of strains showing fluorescence and different colony morphology was performed from the highest dilutions. All bacterial cultures were stored at −20 °C in Tryptic Soy Broth (TSB) supplemented with 20% (v:v) glycerol.

Bacterial characterization was carried out on the basis of colony morphology, Gram stain, oxidase test, production of acids from 1% glucose in Oxidation/Fermentation (OF) basal medium (Hugh and Leifson, 1953), and analysis with the API 20NE biochemical test plus computer software (bioMèrieux S.A., Marcy l'Etoile, France).

2.2. Phytopathogenic fungi and reference bacteria

Fungal phytopathogens used were *Sclerotium rolfsii* Sacc., *Fusarium solani* (from the fungal collection of the Laboratory of Plant–Microbe Interactions, Universidad Nacional de Río Cuarto), and *Alternaria alternata* f. sp. *lycopersici* (kindly supplied by the Laboratory of Mycology, Universidad Nacional de Río Cuarto), all isolated from diseased tomato and pepper plants. Fungi were kept in potato dextrose agar (PDA) plates at room temperature or at 4 °C, and replicated monthly.

Reference bacteria were used in this research. *Pseudomonas fluorescens* CHA0 and *P. aurantiaca* SR1 were grown on KB and 25% Tryptic Soy Agar (TSA). *Serratia marcescens* WF was grown on 25% TSA. *Bradyrhizobium* spp. C 145 and *Sinorhizobium meliloti 3DOh13* were maintained on Yeast Mannitol Agar (Vincent, 1970). All the bacteria were routinely cultured at 28 °C.

2.3. Evaluation of strains for in vitro biological control

2.3.1. Antagonism in dual culture

The fluorescent *Pseudomonas* were tested against *S. rolfsii* Sacc., *A. alternata* and *F. solani* in plate bioassays. *A. alternata* and *F. solani* were cultivated in PDA at 28 °C. Conidia were harvested from the surface of plates by flooding the 10-day-old cultures with 9 ml of sterilized distilled water and gently scraping with a sterilized glass rod; conidial concentration was determined with a Neubauer chamber (Cota et al., 2007). Plates containing the media to be tested (KB, PDA) were prepared. Then, an agar over-layer containing the target fungus, immobilized at a concentration of 10^4–10^5 conidia ml^{-1}, was placed on the medium. The methodology described by Montesinos et al. (1996) was followed in order to prepare the overlay, using 0.7% agar. Four ml of the medium was placed in screw-capped test tubes that, once sterilized, were kept inside of a bath of water at 40 °C. Next, 100 μl of a target conidia suspension was added to each test tube, which were vortexed and the content of each tube was then homogeneously distributed on a plate containing the same culture medium. The bacterial strains tested were sown by gently touching the agar surface with a sterile toothpick, previously inoculated by touching the surface of a single colony. Plates were incubated for 72 h at 28 °C. The degree of inhibition in each medium was determined by measuring the halo around the bacterial strain with no fungal growth. The average of six replicates was considered for the value of the inhibition halo. For screening for potential antagonism against *S. rolfsii* Sacc., mature sclerotia were removed from the surface of 15-day-old cultures with sterile forceps and four were immediately placed around the edges and one in the center of a plate 24 h after the stab-inoculation of four bacterial strains. The experiment was conducted twice.

2.3.2. Mycelial growth inhibition

The bacterial strains were streaked on 1/3 of a Petri plate containing 25% TSA, KB or PDA. A mycelial disc (9 mm diameter) of a 8–15 day-old-culture of an actively growing target fungus was equidistantly placed on the opposite side of the Petri plate 48 h after inoculation of the strain. Plates were incubated for 7 days at 28 °C. The plates with fungal pathogens on one side that were not inoculated with bacterial strains served as controls. For each fungal colony, two diameters, measured at right angles to one another, were averaged to find the mean diameter for that colony. The mean diameter of fungal growth in the presence of each strain was compared to that of the control cultures in order to determine the inhibition percentage. All fungal colony diameters were determined by using three replicates for each strain on each medium. *P. aurantiaca* SR1 (Rosas et al., 2001; Rovera et al., 2008) and *P. fluorescens* CHA0 were used as positive controls.

In addition, each strain was tested on both 25% TSA and KB supplemented with two concentrations of $FeCl_3$ (50 and 100 μM) in order to evaluate the influence of iron on the ability of the strains to control *A. alternata*. Plates were incubated for 7 days at 28 °C. The fungal colony diameter was determined by using three replicates for each strain on each medium. The plates with *A. alternata* on one side that were not inoculated with bacterial strains served as controls. Experiments were conducted twice.

2.3.3. Production of hydrolytic enzymes

Proteolytic activity was detected by inoculating the strains on a medium composed of 1% casein and 2.3% agar dissolved in Castañeda medium (Castañeda-Agulló, 1956). Plates were incubated for 48 h at 28 °C. Casein hydrolysis was detected by the formation of a whitish, opaque halo (coagulated casein) around a translucent area (totally hydrolyzed casein), surrounding the colony. Strains were also tested for its ability to produce extracellular chitinases in a liquid medium; assay medium was prepared with 2% chitin from crab shells (w:v) in tap water (Rojas Avelizapa et al., 1999). *S. marcescens* WF was used as a positive control. Tests were performed twice. To determine cellulolytic activity, carboxymethyl cellulose (CMC) was incorporated at 0.1% into the YEMA–0.2% mannitol agar plates. Colonies were grown for 3 days at 28 °C and